Ultrastructural aspects of the hypanthial epithelium of *Selenicereus grandiflorus* (L.) Britton & Rose (Cactaceae)

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Summary

The epithelium of the basal part of the inside floral tube (hypanthium) of *Selenicereus grandiflorus* (L.) Britton & Rose was investigated with ultrastructural and cytochemical methods, while a Gas Chromatography/Gas Mass analysis was performed on the secretion products of this flower tissue.

Despite its position (frequently occupied by “classical” nectaries in many representatives of Cactaceae) the cells of the investigated epithelium show features that are typical of cells involved prevalently in lipidic production. Lipids are stored in the big vacuole which fills almost the whole cell in the last developmental stage. The secretion is of holocrine type and probably started by the mechanical action of the pollinator itself. However sugars conveying from the underlying parenchyma towards the epithelium and a strong PAS-positive reaction at the vacuolar level was observed. These observations suggest also an important presence of carbohydrates in the last products. The formation of subcircular spaces indicates also an exocrine type of secretion. The results obtained with Gas Chromatography/Gas Mass analysis indicate the production of some phenols, that we link to flower scent.

This is the first report on oils producing flowers among Cactaceae and the first for nocturnal flowers.

Key words: Cactaceae, epithelium, hypanthium, nectar, oil flowers, *Selenicereus*.

Introduction

*Selenicereus grandiflorus* (L.) Britton & Rose is a member of the family Cactaceae. *S. grandiflorus* produces big white tubular flowers that open in the night. These flowers are probably visited by moths like in other representatives of this genus with similar flowers (BARTHLOTT & HUNT 1993; BARTHLOTT et al. 1997).

The flowers of *S. grandiflorus* are formed by tepals and release a strong sweet fragrance at the anthesis. The inferior ovary results from intercalary growth under the perianth, forming a long hypanthium.

In many Cactaceae the nuptial nectaries (senso CASPARY 1848 and VOGEL 1998) are present along the internal side of the floral tube, just above the ovary, that is, along the basal part of the hypanthium (BUXBAUM 1959; BARTHLOTT & HUNT 1993; NASSAR et al. 1997); in these cases a nectar chamber is often present. Pollinators are thus obliged to reach the bottom of the flower to find nectar.

Recently the existence of flowers offering oil and not nectar to their pollinators has been reported. The oils would be provided by glands termed elaiophores (VOGEL 1969; ENDRÉSS 1994; SIMPSON et al. 1977).

Since we did not find a well-localised nectariferous tissue in *S. grandiflorus*, we investigated the features of the internal epithelium along the hypanthial basal part, with the aim to evaluate its activity in relation to pollinators attraction.

Materials and methods

Flowers of *S. grandiflorus* were collected by the authors in the State of Vera Cruz (Mexico) in 1997. Flowers of different length were sampled: 2 cm; 5 cm; 8 cm; 12 cm (one day before anthesis); 15 cm (anthesis).

Pieces of the hypanthium, approximately 2 mm in length, were fixed in a solution of 2.5% glutaraldehyde, adjusted to pH 7.1 with phosphate buffer, postfixed in 2% OsO4 in the

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same buffer, then dehydrated and embedded in epoxy resin (Suur 1969).

Ultrathin sections obtained with a Reichert OM U3 ultramicrotome were stained with uranyl acetate (Griffiths & Grimstone 1960) and subsequently with lead citrate (Reynolds 1963). The observations and recording of images were performed with a Philips EM 300 transmission electron microscope at 80 kV.

The PAS reaction was done according to the procedures described in Hotokin (1948) and Chen et al. (1969) on paraffin-embedded samples.

The secretion product of the hypohyaline epithelium was drawn at the anthesis time by introducing a mixture of 5 cm³ Acetone and 5 cm³ dichloromethane and with mechanical rupture of the cells with a glass rod. The GCMS instrument consisted of a Varian 3400 Gas Chromatograph (Varian, Valvar Creek, USA) and a Varian Saturn 2000 Ion Trap Mass Spectrometer. The chromatographic column was SPB-5 (Supelco, Bellefonte, PA, USA) (30 m × 0.32 mm LD, 0.25 micrometer film thickness).

Gas chromatographic elution temperature programmed as follows: 3 minutes initial isothermal step at 60°C, then up to 320°C in 52 minutes; this final temperature of 320°C is then maintained for 5 minutes, for an overall analysis time of 60 minutes. The injector set temperature was 270°C. Analyses were performed in split mode, at a split ratio of 1:25 with injection volumes of 1 microliter. The transfer line temperature was set at 260°C.

Mass spectra were acquired in Electron Impact mode, with a mass range of 35–450 amu (34 mlz of background mass) and a scan speed of 1 scan per second. The ion trap was kept at a temperature of 220°C, with a multiplier voltage of 1650.

Substances eluted from the column were recognized by the injection of standards, the use of mass spectra data, and by comparison with the collection of mass spectra in software libraries (NIST/EPA/NIH 1992).

**Figure legends for the following tables**

**Key of labelings:** p = plastids; v = vacuole; n = nucleus; m = mitochondria; w = wall; er = endoplasmic reticulum; re = rough endoplasmic reticulum; d = dictyosomes; e = epithelial cells; pe = parenchymatous cells; e = eider cells

Fig. 1. Flower of Selicentrurus grandiflorus 2 cm long. The cells of the hypohyaline epithelium are not yet well differentiated. A visible electron dense layer is present on the outer tangential wall. Some small vacuoles are distributed in the cytoplasm around the big active nucleus. The plastids have scarce thylakoids. bar = 1 μ

Fig. 2. Flower 2 cm long: epithelial cell. The outer tangential wall has assumed a convex shape, increased its thickness and assumed an indented profile. Microtubules (arrow) appear close to the outer tangential wall. bar = 0.5 μ

Fig. 3. Flower 2 cm long: epithelial cells. The vacuoles are fusing. Single starch grains appear in the plastids. bar = 2 μ.

Fig. 3a. Flower 2 cm long: epithelial cells. Single starch grains (asterisk) appear in the plastids. bar = 0.5 μ

Fig. 4. Flower 2 cm long: epithelial cell. Bundles of parallel microfilaments (arrow) are visible, arranged outside a tract of the tonoplast of the main central vacuole. bar = 0.5 μ

Fig. 5. Flower 5 cm long. The epithelial cells have a more elongated shape in the radial axis. The radial walls are separating starting from the distal side. Almost all the cellular volume is occupied by a big vacuole. In the perivascular cytoplasm (arrow) of the central vacuole filled with amorphous and osmiophilic material appear. bar = 5 μ

Fig. 6. Flower 5 cm long: epithelial cell. Detail of Fig. 5. The central vacuole discharging their content is in. Plastids own remarkable starch grains (asterisk), some showing a normal aspect and others dissolving. Plasmodesmata across the walls connect the epithelial cells. bar = 1 μ

Fig. 7. Flower 8 cm long: epithelial cell. The electron dense material made of notable aggregates, approximately roundish in shape, appears. In the vascular sap a scattered flocculent substance becomes visible. Big starch grains (asterisk) are visible both in the plastids of the epithelial cells and in those of the parenchymatous cells. bar = 2 μ

Fig. 8. Flower 8 cm long: epithelial cell. The electron dense material along the inner side of the tonoplast tends to form a continuous layer adhering to the tonoplast. The flocculent substance is homogeneously distributed in the vacuole. Starch granules (asterisk) in the plastids. bar = 1 μ

Fig. 9. Flower 8 cm long. The cells of the parenchyma which subtends the epithelium show a rich and active cytoplasm where plastids are numerous and with big starch grains (asterisk). bar = 2 μ

Fig. 10. Flower 12 cm long (a day before the anthesis). Plasmodesmata (arrow) across the wall between an epithelial cell and a parenchymatous cell. bar = 1 μ

Fig. 11. Flower 12 cm long (a day before the anthesis). In the epithelial cells (cross section) the vacuolar osmiophilic material appears to be distributed in small and roundish bodies, dispersed in the flocculent vacuolar sap. Inside the plastids starch persists. In the cells of the parenchyma, far smaller than those of the epithelium, an important storage of plastidial starch is removable. bar = 5 μ

Fig. 12. Flower 12 cm long (a day before the anthesis). The cuticle of the epithelial cells rises forming subecticular spaces "galls" (arrow). Starch grains (asterisk) are present in the sable peripherical cytoplasmic belt. bar = 2 μ

Fig. 13. Flower 15 cm long (anthesis time). The epithelial cells have lost their symplastic connections with the underlying parenchyma. The vacuole the electron dense material is now distributed in big aggregates. The cells of the parenchyma have lost their plastidial starch. bar = 10 μ

Fig. 14. Flower 15 cm long (anthesis time). The cytoplasm of the epithelial cells is reduced to a few islands where each component assumes a strong osmiophility. It is hardly possible to recognize some mitochondria and ER elements. bar = 0.5 μ
Results

Ultrastuctural observations. - When the flower was 2 cm long (that is about 1/7 of its final dimension) cells that form the future hypanthial epithelium looked similar to those of the underlying layers (Fig. 1).

In cross section the epithelial cells were of an almost rectangular shape with a subulate electron dense layer on the surface of the outer tangential wall. In the cytoplasm fusion of the small vacuoles were evident (Fig. 1). The nucleus was euchromatic and spherical and occupied a large part of the cellular volume; it contained a spongy nucleolus. The plastids were elongated and of irregular shape, thylakoids were scarce and often in division. Mitochondria (Fig. 1) were small, with few cristae and a ruffled matrix. Many ribosomes were present. RER, SER and dictyosomes were evident and homogeneously distributed. Plasmodesmata crossed the radial and the internal tangential walls (Fig. 1).

Somewhat later in the same stage (2 cm long flowers), microtubules arranged in parallel arrays appeared close to the outer tangential wall (Fig. 2). This situation was observed when this wall showed a convex shape, increased thickness, and an indented profile on the external side (Fig. 2, 3). Single starch grains appeared in the plastids (Fig. 3a). Bundles of parallel microfilaments were visible, arranged outside some tracts of the tonoplast profile (Fig. 4).

In the floral tubes of 5 and 8 cm of length, the epithelial cells reached a more elongated shape in the radial axis and separated the radial walls starting from the distal side (Fig. 5). These cells maintained the symplastic connections (plasmodesmata) on the basal part of the radial walls (Fig. 6) and with the underlying parenchyma. The nucleus was more often located at the cellular basin. The ER, which had developed widely and was distributed throughout the whole cytoplasm, showed well localized swellings in cisternae of different shapes and dimension (Fig. 5, 6). These cisternae, which contained an amorphous and osmiophilic material, appeared to join the central vacuole (Fig. 6). Close to them many mitochondria with well developed cristae were visible. Plastids owned big starch grains, some of which showing a normal aspect and others dissolving (Fig. 6).

The same developmental stages were observed in flowers of 5 and 8 cm of tube length. The following cytoplasmatic modifications were evident: the enlarged central vacuole constrained the cytoplasm to a peripheral layer (Fig. 7); on the inner side of the tonoplast an electron dense material made of notable aggregates, approximately roundish in shape, or fused in a continuous layer along the whole vacuolar perimeter, appeared (Fig. 7, 8). In the vacular content a scattered flocculent substance, often adhering to the surface of the electron dense material appeared. Narrow and long ER profiles became visible along the tonoplast in a ribosomes-rich cytoplasm.

In cells of the underlying parenchyma the abundant supplies of plastidial starch were evident (Fig. 9).

A day before anthesis the flower tube was about 12 cm long. The cells of the epithelium had increased their dimension notably. They often lost the contact between them (the middle lamellae had disappeared) but maintained the connection through the plasmodesmatal bridges with the underlying parenchyma (Fig. 10). The osmiophilic vacuolar material appeared to be distributed in the flocculent content as small and roundish bodies, sometimes coalescing (Fig. 11). Inside the plastids some big starch grains persisted. The cuticle of the epithelial cells raised forming "galls" (subcellular spaces) (Fig. 12).

At this stage long ER profiles were rare. In the cells of the parenchyma, far smaller than those of the epithelium, starch persisted (Fig. 11).

Around anthesis, when the flower tube is about 15 cm long, the epithelial cells had lost their nuclei and the symplastic connections with the parenchyma. Inside the vacuole the electron dense material was now distributed in big aggregates (Fig. 13). The cytoplasm was reduced to a very subtle and very osmiophilic belt where it was still possible to recognize some mitochondria and plastids (sometimes with starch) and short ER tracts (Fig. 14). The parenchymatic cells had lost their plastidial stach (Fig. 15).

Cytocchemistry results. - The epithelial cells of 15 cm long tubes (at the anthesis) stained strongly with PAS reaction, prevalently in the vacuole (Fig. 15).

Gas chromatograph/mass spectrometry results. - A preliminary GC/MS of the Dihexomethane extract is shown in Fig. 16. The gas chromatographic profile shows the separation of 22 substances and the chemical structures of two compounds identified. These are guaiacol (2-methoxy-phenol) and allylsyringol (2,6-dimethoxy-4-propenyl phenol). Such phenolic compounds can be attributed to the essential oil fraction of the flower (ADAMS, 1995). The late eluting compounds (group 3 in Fig. 16) were tentatively recognized by mass spectra as long chain hydrocarbons, alcohols and ethers.

Discussion

The development of the hypanthial epithelium of S. grandiflora can be divided in three successive stages, i.e. (1) differentiation, (2) maturation, (3) secretion. Differentiation occurred at a flower bud length of 3 cm. In this stage the microtubules observed along the walls, particularly those arranged in parallel to the outer
tangential wall, might be responsible for the cellular shape modifications, involving both cell elongation and the attainment of a convex form. Microtubules in plants are in general linked to the spatial control of cell expansion (Nick 1998). Particularly, microtubules disposed in arrays adjacent to the plasma membrane are understood to control the direction of cellulose deposition and thus the axis of cell expansion (Green 1980; Williamson 1991; Cyr 1994; Nick 1998; Vaughn & Harper 1998).

The stage of epithelium cellular maturation occurs when the flower bud grows from about 5 to 8 cm. Asynchronous developmental steps can be observed. The elongated shape and the position of the nucleus at the base of the epithelial cells were assumed as secretory features. The microfilaments observable along tracts of the tonoplast might be involved in the vacuolar shape modifications observed; increase in dimension and fusion with other vacuoles. The maturation stage ends with the enlargement of ER elements that are combined with secretion into the vacuole.

Plasmodesmata connecting the epithelial cells assure a uniform development of these cells. Plasmodesmata connecting epithelial cells to the underlying parenchyma indicate the necessity of an exchange of substances among the two tissues.

The secretion stage begins when the swollen SER fuses with the tonoplast and conveys its osmophilic content into the vacuole.

Changes in the vacuolar content. - After the formation of the large central vacuole the localized SER swellings indicate the production of a lipidic material destined to be deposited in the vacuole. We analysed the secretion product drawn at the anthesis time to identify the components. With GC/MS analysis we identified two hydrophobic compounds, guaiacol and allyl-syringol, together with late eluting compounds (group 3 in Fig. 16), that were tentatively recognised by mass spectra as long chain hydrocarbons, alcohols and ethers.

These compounds are typical constituents of waxes (long chain hydrocarbons), flavours and essential oils (long chain alcohols and ethers) (Adam 1995). These classes of compounds are widespread among plants. Especially flavours and essential oils are known to be related to the typical attraction mechanism of plants.

After the fusion of the SER with the tonoplast the lipidic content enters the vacuole occupying a peripheral position in contact with the tonoplast, eventually forming a thick continuous layer. The appearance of the lipid aggregates can be attributed to their hydrophobic properties which make them unable to dissolve in the vacuolar sap. Afterwards the vacuole appears to contain an abundant flocculent material. By mixing the lipidic content, the flocculent material causes the emulsion phase to dissolve. As a matter of fact this material is particularly abundant at the interface between lipids and vacuolar sap.

The presence of an emulsion implicates the dissolution of the continuous lipidic layer which acted as a barrier against the passage of new hydrophilic secretion products. Consequently the normal permeability of the tonoplast was recovered.

The migration of hydrophilic products into the vacuole leads to the rupture of the emulsion. As a matter of fact, at the anthesis time, the lipidic masses form aggregates of larger bodies, which mainly occupy a peripheral position. The PAS positivity of the vacuolar content indicates that the hydrophilic substances entered into the vacuole and carbohydrates may be responsible for the rupture of the emulsion even if also unsaturated fatty acids may react with Periodic Acid (Chayan et al. 1969). The presence of fatty acids, both saturated and unsaturated can be deduced from the GC/MS analysis. The secretion products of the aleaephores of the oil flowers have been identified as fatty acids, mostly acetoxy fatty acids (Seigler et al. 1978).

During the secretion stage of the epithelial cells, it is possible to observe the formation of subcubicular spaces (galls). The presence of a subcubicular space with an electron translucent content has previously been indicated for gland cells by Schaepe (1969b, 1974), where it marks the beginning of secretion. Such features indicated an exocrine secretion (Schaepe 1969a, 1974). The phenols (detected with GC/MS analysis) might be released through the cuticle. They are probably components of the floral fragrance.

At the completion of the epithelium development the vacuole occupies almost the whole cell volume. The secretion product is released in a holocrine way through mechanical breakdown of the cells.

The persistence of mitochondria throughout development has often been observed in tissues destined to degradation as in the anther tapetum of Tillandsia albida (Brighina & Papini 1993) and of Antirrhinum majus (Lombardo & Carraro 1976). This fact is also observed in apoptosis in animals, at least in the early phases, as in cat hemolymphatic cells (Rokko et al. 1996). The persistence of mitochondria indicates the continuous energy demand, suggesting that the final events of the epithelial development described here are not a simple necrosis, but comparable to programmed cell death (Sensi Pennell & Lamb 1997; Papini et al. 1999).

The position of the studied epithelium (at the level of the lower part of the hypanium) in the flower of S. grandiflorus is the same indicated for the nectaries of many Cactaceae (Buxbaum 1959; Bartlett & Hunt 1993; Nassar et al. 1997), but a nectar chamber is not present.

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Fig. 15. Flowers 15 cm long (anthesis time). LM image. The epithelial cells stain strongly with PAS reaction. bar = 100 μ

Fig. 16. GC/MS results. The gas chromatographic profile of the extract. Structures have been reported for compounds 1 and 2: guaiacol and allyl-syringol, respectively. Later eluting compounds (group 3) were tentatively identified by Mass Spectra as long chain hydrocarbons, alcohols and ethers.

We conclude that moths are the pollinators of the flowers of *S. grandiflorus*. That because the epidermis, which is located at the basis of a long floral tube, release a sweet scent. Moths are the pollinators indicated in other species of *Selemicereus* with analogous floral features (BARTHLOTT & HUNT 1993; BARTHLOTT et al. 1997).

The investigated epithelial cells did not show the features that were indicated as typical for nectaries secreting sugars by FERR (1988) and FIGUEIREDO & PAIS (1992). Cell wall ingrowths were not observed in *Selemicereus*, but this feature is absent also in some other nectaries (FIGUEIREDO & PAIS 1992). The hypanthial epithelial cells of *S. grandiflorus* are devoted to lipids production, while the type of secretion appears to be at last holoceric (sensu SCHNEPP 1969a). The rupture of the epithelial cells and hence the holoceric secretion might be induced by the action of the pollinator itself.

Because of the production of lipids, the hypanthial epithelium of *S. grandiflorus* can be considered an elaiophore (tissue devoted to oil production for pollinators) (VÖGEL 1974). The present work is the first report of elaiophores in the Cactaceae family. Elaiophores have

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been found until now in a few angiosperms families: Malpighiaceae, Krameriaeeae, Cucurbitaceae, Primulaceae, Solanaceae, Scorpiophurariaeae, Iridaceae, Orchidaceae, prevailingy in New World species (ENDRESS 1994). Elaiophores are linked to some specialized bees (VOGEL 1974; SIMPSON et al. 1977; SIRASI & COCCUCCI 1999) which use the oils produced together with pollen for larval food supply or for the construction of the nest cell lining (VOGEL & COCCUCCI 1995). However S. grandiflorus has nocturnal flowers, therefore bees might not be its pollinators. Also the pathway of secretion may be different: in the other elaiophores it is thought that the bees, equipped with a scraper of bristles force the oils through pores in the cuticle (SIMPSON & NIFFE 1981). However in S. grandiflorus the pores in the cuticle are evident, so we may suggest a simple rupture of the epidermal cells due to a less specialized mechanical action.

A symplastic transport of sugars from the underlying parenchyma towards the epidermis is evident, since plastids of the parenchyma store an increasing quantity of starch until the anthesis. At this stage starch grains were no longer visible in the parenchyma. The same starch accumulation by plastids has been observed in the parenchyma beneath the nectariferous tissue of Limodorum alatum (FIGUREDIO & PAS 1992). The plastids present in parenchyma and epidermis of Selenteeereus have probably the role of transitory carbohydaraes storage since the activity of chloroplasts of petals is normally low (WEISS et al. 1988; MATAK et al. 1998). Disintegration and mobilization of the starch found in the secretory tissue and water from the vacuoles serve as sources of nectar: the appearance of nectar occurs simultaneously with starch degradation in various nectaries (GAFFAL et al. 1998). A symplastic route of nectar components has been suggested by FINDLAY (1988) and FIGUREDIO & PAS (1992).

The simultaneous presence of lipids and sugars in many "oil" flowers are indicated by BAKER (1978) and PERCIVAL (1961), while in Krameria and Stigmaphyllum no trace of sugars was found (SIMPSON & NIFFE 1981).

Since subcuticular spaces have been observed also in S. grandiflorus, with ER profiles along the plasma membrane similar to those of various nectaries (FAHIN 1988; FIGUREDIO & PAS 1992), sugars coming from the parenchyma are probably released via a secretory secretion. Hence the structure of S. grandiflorus would provide its pollinators both with fats and sugars.

SIMPSON & NIFFE (1981) and VOGEL (1998) defined the characters respectively of the epidermal elaiophores and of the epidermal nectaries considering their positions and morphological aspects. For its position in the flower and ultrastructural features the hypoeppithelial of S. grandiflorus can be added to both of the two functional groups.

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